

(2015) *Journal of Photochemistry and Photobiology B: Biology*

***In vivo* reconstitution of a homodimeric cytochrome b_{559} like structure: The role of the N-terminus α -subunit from *Synechocystis* sp. PCC 6803.**

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Keywords: alpha-helix; cytochrome; membrane; photosynthesis; chimeric protein; protein assembly.

Abbreviations: Abs, absorbance; BCA, bicinchoninic acid; Cyt, cytochrome; D1 and D2, the two core-subunits of the PSII reaction center; *E.*, *Escherichia*; *Eh*, ambient redox potential; *Em*, midpoint redox potential; EPR, electron paramagnetic resonance; IPTG, isopropyl β -D-1-thiogalactopyranoside; MBP, maltose-binding protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PSII, photosystem II; Q_B , secondary quinone acceptor of the reaction center of PSII; SDS, sodium dodecyl sulfate.

Abstract

The cytochrome *b*₅₅₉ is a heme-bridged heterodimeric protein with two subunits, α and β . Both subunits from *Synechocystis* sp. PCC 6803 have previously been cloned and overexpressed in *Escherichia coli* and *in vivo* reconstitution experiments have been carried out. The formation of homodimers in the bacterial membrane with endogenous heme was only observed in the case of the β -subunit (β/β) but not with the full length α -subunit. In the present work, reconstitution of a homodimer (α/α) cytochrome *b*₅₅₉ like structure was possible using a chimeric N-terminus α -subunit truncated before the amino acid isoleucine 17, eliminating completely a short amphipathic α -helix that lays on the surface of the membrane. Overexpression and *in vivo* reconstitution in the bacteria was clearly demonstrated by the brownish color of the culture pellet and the use of a commercial monoclonal antibody against the fusion protein carrier, the maltoside binding protein, and polyclonal antibodies against a synthetic peptide of the α -subunit from *Thermosynechococcus elongatus*. Moreover, a simple partial purification after membrane solubilization with Triton X-100 confirmed that the overexpressed protein complex corresponded with the maltoside binding protein-chimeric α -subunit cytochrome *b*₅₅₉ like structure. The features of the new structure were determined by UV-Vis, electron paramagnetic resonance and redox potentiometric techniques. Ribbon representations of all possible structures are also shown to better understand the mechanism of the cytochrome *b*₅₅₉ maturation in the bacterial cytoplasmic membrane.

1. Introduction

The photosystem II (PSII) is a membrane-bound supercomplex, which function is to extract electrons and protons from water at the lumen side and reduce plastoquinones at the stroma side to generate electron transfer and proton gradient across the thylakoid membranes. Dioxygen is produced as a byproduct of that process, which is liberated to the atmosphere. The PSII supercomplex is made of many protein subunits, most of them inserted within the thylakoid membranes with α -helix secondary structure conformation [1,2]. The natural cytochrome (Cyt) *b*₅₅₉ is a heterodimeric (α/β) metalloprotein that co-purify with the D1-D2 heterodimer (called D1-D2-Cyt *b*₅₅₉ complex) [3,4]. Both cytochrome protein subunits are of chloroplastic origin (α and β subunits are the products of the *psbE* and *psbF* genes, respectively), and span the

thylakoid membrane with a single α -helix. Each subunit contains a histidine that coordinates the heme group towards the stromal side of the thylakoid membrane [5,6] making a hexacoordinate low spin iron [7-9].

Cytochrome b_{559} shows two unique features with respect to other cytochrome b -type proteins. Indeed, most of those cytochromes are made of a single polypeptide and their midpoint redox potentials (E_m) vary between -100 and +100 mV [10-12]. However, the Cyt b_{559} is a heterodimeric (α/β) protein [3,4] and it can reach an unusually high redox potential up to +400 mV that can widely vary depending on sample preparations and mutations [10-15]. These two properties make this metalloprotein more elusive and difficult to characterize, and establish its functional role.

The function of the Cyt b_{559} is not well resolved yet but the most accepted hypothesis nowadays considers that it is involved in the photoinhibition/repair cycle of the PSII [12,15-18]. Accordingly, this cytochrome would accept electrons from an overreduced acceptor side (acceptor side photoinhibition) or deliver electrons to a deficient electron donor side (donor side photoinhibition) of the PSII. The secondary plastoquinone (Q_B) is most probably the best candidate to donate electrons to the Cyt b_{559} and the β -carotene of the D2 protein to accept electrons from the Cyt b_{559} [12,15,16,18]. Indeed, the heme moiety of this cytochrome is strategically located between the secondary Q_B and the β -carotene in the crystal structure [2,6]. For such a role, the Cyt b_{559} has to change easily its redox state from reduced high potential to oxidized low potential forms. Such redox variability is actually observed experimentally in many different preparations of PSII and mutants [11,12,14,15,18] though the underline molecular mechanisms for those changes are yet unknown.

In a previous publication [19], we described the *in vitro* reconstitution of a Cyt b_{559} like structure using the recombinant subunits from the plant sugar beet overexpressed in *Escherichia (E.) coli* and partially purified from inclusion bodies. Thus homodimeric and heterodimeric Cyt b_{559} like structures were reconstituted using commercial heme with a yield somewhat higher for the heterodimer (α/β) than the homodimers (α/α ; β/β) [19]. *In vivo* reconstitution was also possible within the *E. coli* cytoplasmic membrane overexpressing only the β -subunit from the cyanobacterium *Synechocystis sp.* PCC 6803, confirming previous *in vitro* results from chemically

synthesized β -subunit and free heme [20] and *in vivo* results in recombinant *E. coli* cells [21]. However, *in vivo* reconstitution overexpressing only the cyanobacterial α -subunit was unsuccessful, despite that subunit was actually incorporated and stabilized within the bacterial membrane [19,22]. Thus the heme group was apparently not necessary for the incorporation and stabilization of the Cyt *b*₅₅₉ α -subunit within the membrane. No logical explanation for this result was found at that time, considering the high structural homology between α - and β -subunits. However, a closer examination of the published crystal structure of the PSII core complex from cyanobacteria [6,23] revealed the presence of a short amphipathic α -helix at the out-of-membrane N-terminus of the α -subunit just on top of the heme group niche, which is not observed in the N-terminus of the β -subunit. The presence of two α -helices on the membrane surface on top of the heme group niche of a (α/α) homodimer would suggest the existence of a possible steric hindrance for heme incorporation and its coordination with the two histidines during the holoprotein maturation. To confirm this hypothesis, a chimeric N-terminus *psbE* gene was generated and incorporated into *E. coli* competent cells, and the resultant recombinant bacterial cytoplasmic membrane fragments were analyzed by SDS-PAGE, immunoblot, UV-Vis, electron paramagnetic resonance (EPR) and redox titration potentiometry techniques.

2. Materials and Methods

2.1. Cloning and expression in *E. coli* of full length and truncated Cyt *b*₅₅₉ α -subunit from *Synechocystis* sp. PCC 8603

The full length *psbE* gene from the cyanobacterium *Synechocystis* sp. PCC 6803 was cloned and overexpressed as in [19,22]. A chimeric Cyt *b*₅₅₉ α -subunit was generated by removing the first sixteen amino acid residues of the N-terminus, thus the new chimeric protein sequence began at the isoleucine 17 (called thereafter Ssub α I17 or α I17). To achieve it, the gene was amplified by PCR using genomic DNA with specific primers containing Bam HI site forward primer and Hind III site reverse primer. Then the gene was ligated to the expression vector pMAL-c2X, and the sequence was confirmed by DNA sequencing. The construct will originate a fusion protein containing the maltose binding protein (MBP), a polylinker, and the truncated N-terminus Cyt *b*₅₅₉ α -subunit. Note that in the *malE* gene of the commercial expression vector pMAL-c2X, a signal sequence leading to periplasmic expression of the final fusion protein has been

deleted (for more information see Instruction Manual pMAL System, New England Biolabs). As a consequence, the MBP protein cannot cross the cytoplasmic membrane to the periplasmic space and thus remains in the cytoplasm. The topology of the MBP protein expressed with these vector series has been examined extensively and unambiguously demonstrated that is located in the periplasmic space when the signal sequence is complete (pMAL-p2) but it appears in the cytoplasm when the signal sequence has the above mentioned deletion (pMAL-c2X) [21,24-26]. According to this information and the structure of our construction, the heme group of the chimeric homodimeric (α/α) Cyt b_{559} like structure has to be located towards the cytoplasmic side of the bacterial membrane. Two more chimeric constructs were obtained at the N-terminus following the same methodology, one without the first eight residues (chimeric Ssub α P9 that preserves the short amphipathic α -helix) and the other without the first twelve residues (chimeric Ssub α I13 removing about half of the short α -helix).

Protein overexpression in *E. coli* TB1 competent cells was described in detail in [19,22]. Essentially, the overexpression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 37 °C for 3 h or 1 mM IPTG at 18 °C for 5 h. Cytoplasmic membrane fragments were obtained according to the protocol published in the aforementioned paper. Bacterial cells were broken by sonication and the crude extract was clarified by centrifugation at 10,000 x g for 10 min. The supernatant was centrifuged at 100,000 x g for 45 min, and the resultant pellet, resuspended in 50 mM HEPES, pH 7.5, corresponded to the cytoplasmic membrane fragments, the material used in the present study.

Total protein content was determined using bicinchoninic acid (BCA) reagent colorimetric method (Pierce Thermo Scientific, Rockford, IL USA). Protein separation was done in SDS-PAGE [12% (w/v) acrylamide, 4 M urea] after denaturation in boiling water bath for 5 min. Then proteins were revealed by Coomassie brilliant blue staining or blotted onto nitrocellulose membrane with a transfer system (Trans-Blot Turbo, BioRad). Membranes were sequentially incubated with a commercial mouse monoclonal anti-MBP (New England Biolabs, County Road, Ipswich, MA USA) or mouse polyclonal antibodies against a synthetic peptide (TPRPDSYYAQEQRS) of the C-terminus α -subunit from the cyanobacterium *Thermosynechococcus (T.) elongatus*. Secondary antibodies were goat antimouse IgG, coupled to horseradish peroxidase. Blots were finally colorimetrically developed with H₂O₂ and 4-chloro-1-naphthol.

2.2. Cytoplasmic membrane fragment treatment with NaBr or TRIS-HCl

To remove potential peripheral membrane proteins, cytoplasmic membrane samples were incubated with 2 M NaBr or 50 mM TRIS-HCl, pH 8.0, for 30 min at 4 °C with gentle swirling. The process was stopped by adding equal volume of cold water, and the suspension was ultracentrifuged at 100,000 x g during 45 min to separate the insoluble membrane fraction as pellet from the supernatant, where the removed soluble proteins were recovered. The treatment was repeated once in the same conditions.

2.3. Protein purification

The MBP-chimeric SsubaI17 fusion protein was partially purified from cytoplasmic membranes previously treated with 50 mM TRIS-HCl, pH 8.0, as explained above. Treated membranes were solubilized with 1% (w/v) Triton X-100 in 50 mM HEPES, pH 7.5, at 4 °C during 30 min with gentle swirling. The resultant suspension was ultracentrifuged at 150,000 x g during 45 min. The supernatant containing the solubilized material was separated from the pellet for further analysis. The pellet was resuspended in buffer 50 mM HEPES, pH 7.5.

2.4. Absorption spectra

UV-Vis absorption spectra in the 500-600 nm were obtained at room temperature with a Beckman DU 640 spectrophotometer (Beckman Coulter, Brea, CA USA) using 1-cm pathlength cuvette. Samples were used as obtained as blank and then reduced with 10 mM sodium dithionite, giving the reduced *minus* oxidized differential absorption spectra directly. Previous blank oxidation with potassium ferricyanide did not result in a higher intensity of the spectral bands, indicating that the cytochrome was fully oxidized as obtained in the preparation. A differential extinction coefficient ($\epsilon_{559.5 \text{ nm - isosbestic point}} = 17.5 \text{ mM}^{-1} \text{ cm}^{-1}$) [8] of the Cyt b_{559} spectral α -band was used to determine the concentration of the cytochrome in the preparations.

2.5. Electron paramagnetic resonance (EPR)

EPR spectra were recorded with a Bruker ELEXYS E580 spectrometer (Bruker, Karlsruhe, Germany) working at the X-band (frequency around 9.7 GHz). Typical spectra were taken at low temperature (12K) by using an Oxford CF935 liquid helium continuous-flow cryostat (Oxford Instruments, Eynsham, UK). For measurements,

microwave power was kept low enough in order to avoid saturation. Typical measuring conditions were: microwave power, 2×10^{-2} mW; modulation amplitude, 0.2 mT.

2.6. Redox potential measurements

Potentiometric titrations were carried out basically as described in [15,19]. For titrations, samples were suspended in 2.5 mL buffer containing 50 mM MES-NaOH, pH 6.5, and the following redox mediators: 10 μ M diaminodurool ($E'_{m7} = +260$ mV), 10 μ M 2,5-dimethyl-*p*-benzoquinone ($E'_{m7} = +180$ mV), 10 μ M *o*-naphtoquinone ($E'_{m7} = +145$ mV), 2.5 μ M N-methyl-phenazonium methosulfate ($E'_{m7} = +80$ mV) and 10 μ M duroquinone ($E'_{m7} = +5$ mV). Experiments were done at 20 °C under argon atmosphere and continuous stirring. Reductive titrations were performed by first oxidizing with 25 μ M potassium ferricyanide and then reducing it stepwise with small aliquots of 50 mM sodium dithionite. After addition of sodium dithionite, the absorption spectrum between 500-600 nm and the redox potential of the solution were simultaneously recorded by using, respectively, a Hewlett-Packard 8452A diode array UV-Vis spectrophotometer and a Metrohm potentiometer (Metrohm Ltd., Herisau, Switzerland) provided with a combined Pt-Ag/AgCl microelectrode (Microelectrodes Inc, Bedford, NH USA), previously calibrated against a saturated solution of quinhydrone ($E'_{m7} = +280$ mV at 20 °C). Differential absorption spectra of Cyt *b*₅₅₉ recorded at each solution redox potential (E_h) were obtained by subtracting the absolute spectra recorded at each E_h during titrations from the spectra of the fully oxidized cytochrome. The absorbance difference at 559 nm *minus* 570 nm obtained from these spectra was converted into percentages of reduced cytochrome and plotted *versus* E_h . The midpoint redox potential (E_m) values were then determined by fitting the Nernst equation for one-electron carrier ($n = 1$) with 1 or 2 components as needed to the plots, and using a non-linear curve-fitting option of the Origin 6.0 software (Microcal Software, Piscataway, NJ USA).

2.7. Protein modeling

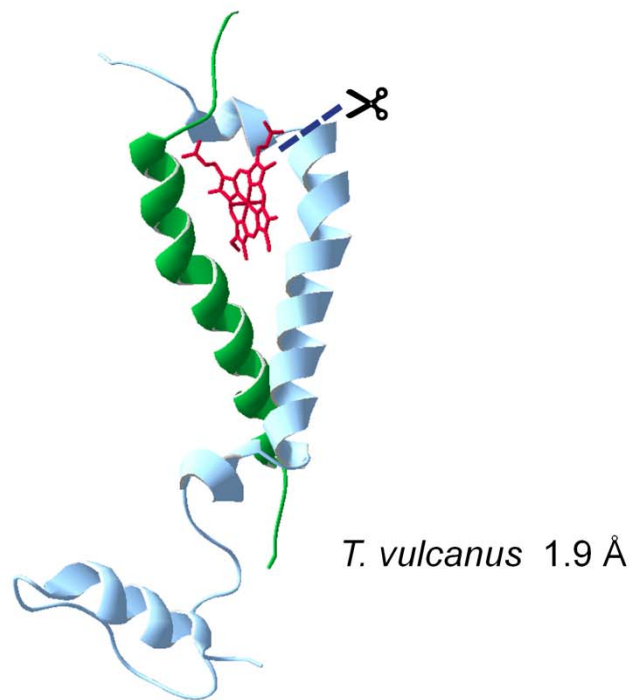
Sequence alignment of the α - and β -subunits of Cyt *b*₅₅₉ (corresponding to E and F chains of the X-ray structure of *Thermosynechococcus* (*T.*) *vulcanus* PSII core: pdb id = 3ARC) shows that the transmembrane α -helix from each subunit that sandwiches the heme group contains 5 conserved residues (α -subunit R18, H23, P28, F31 and G34, and β -subunit R19, H24, P29, F32 and G35). Inspection of the X-ray structure indicates that those conserved residues contribute as follows to the symmetric arrangement of the

helices around the heme. The histidine and arginine residues play key roles in heme binding by coordinating the heme Fe and forming salt bridges with the heme propionic acid side chains, respectively. The small size of the glycine residues allows a close approximation of the two helices at their C-termini, which is reinforced by interhelical contacts between the glycines and phenylalanines. Finally, the prolines force a kink in the helices that allows their better approximation to the intercalated heme. The modeling of the (α/α) dimer was focused in those conserved residues. First, the X-ray structure of the α -subunit was superimposed onto the structure of the β -subunit with *Swiss-pdb viewer* [27], using the *fit from selection* choice so that the five conserved residues of the two subunits were optimally superimposed (overall RMSD = 0.87 Å, or 0.25 Å if the more flexible R residue is excluded). Then a new PDB file was built by combining the coordinates of the original α -subunit in 3ARC with those of the α -subunit previously superimposed onto the β -subunit. In this new file the conserved histidine and arginine residues are correctly positioned to interact with the heme group, and the backbone of the modeled α -subunit transmembrane helix closely follows that of the entire original β -subunit helix in the natural Cyt *b₅₅₉*. The model was energy minimized with the Gromos96 [28] implementation in *Swiss-pdb viewer* until the computed energy of all side chains reached low values indicative of a lack of clashes. Finally, both the Cyt *b₅₅₉* heterodimer (pdb id = 3ARC) and the energy-minimized model of the (α/α) dimer were analyzed with PROCHECK [29]. The various quality scores obtained for the two structures were similar. The model of the truncated (α/α) dimer is based on that of the full length dimer. The model of the (β/β) dimer was done in an analogous manner to that of the (α/α) dimer.

3. Results

3.1. Overexpression of different fusion protein

A ribbon representation of the native Cyt *b₅₅₉* structure obtained from the crystal coordinates of the PSII core complex from the cyanobacterium *T. vulcanus* at a resolution of 1.9 Å [23] is depicted in the Fig.1. This also shows the truncated N-terminus sequence (shade grey) to obtain the chimeric α -subunit from *Synechocystis sp.* PCC 6803 used to transform *E. coli* TB1 competent cells. Since several attempts to overexpress the α -subunit without the MBP protein were unsuccessful, we introduced that protein carrier in the plasmid construction obtaining a good overexpression of the



Suba *Synechocystis* PCC 6803

MSGTTGERPFSDIVTSI RYWVIHSITIPMLFIAGWLFVSTGL
AYDAFGTTPRPDEYFTQTRQELPILQERYDINQEIQEFNQ

Chimeric fusion protein

MBP C-terminus polylinker chimeric I17 Ssuba

...GRQTVDEALKDAQTNSSSSNNNNNNNNNNNLGIEGRISEFGSIRYWVIHSITIPM...

Fig.1: Structure of the natural heterodimeric (α/β) Cyt b_{559} from cyanobacteria. Upper part, ribbon diagram according to the X-ray crystal structure from *T. vulcanus* at 1.9 Å resolution: pdb id = 3ARC showing the place where the N-terminus was cut. Lower part: deduced amino acid sequence of the full length α -subunit from *Synechocystis* sp. PCC 6803 where the removed N-terminus part appears in shade grey; resultant chimeric fusion protein (...MBP-polylinker-chimeric α -subunit...).

MBP- α -subunit fusion proteins. Pellets from bacterial cultures transformed with the MBP alone (Fig. 2a, MBP) grown at 37 °C were colorless as expected. In the overexpression of the full length α -subunit at 37 °C, *E. coli* optimal growth temperature, the bacterial pellet was also colorless and no MBP-Ssub α fusion protein was observed using SDS-PAGE analysis [22]. We do not have a clear explanation for this result but it could be due to a lesser fusion protein expression, lower protein stability, more prone to protease digestion character, and so forth. To overcome the problem, several growth conditions were tested in this work, and induction at 18 °C for 5 h were the best conditions found to produce a stable fusion protein. With these new growth conditions, the MBP-full length Ssub α fusion protein clearly appeared in the SDS-PAGE at the expected molecular weight, but the pellet from those bacterial cultures still remained colorless (Fig. 2a, α), indicating that no reconstitution of a Cyt *b*₅₅₉ like structure was obtained. However, cells with the MBP-chimeric α -subunit grown both at 18 °C (Fig. 2a, α I17 18 °C) or 37 °C (Fig. 2a, α I17 37 °C) showed a distinct brownish color, indicating the reconstitution of a chimeric homodimeric (α/α) Cyt *b*₅₅₉ like structure in *E. coli*. Figure 2b shows the protein analysis by SDS-PAGE of the membranes obtained from the different cell cultures with empty vector (lane 1), MBP protein alone (lane 2), MBP-full length Ssub α fusion protein (lane 3), and MBP-chimeric Ssub α I17 fusion protein from cells grown at 18 °C (lane 4) or 37 °C (lane 5). Since the chimeric fusion protein appeared somewhat more abundant from cells grown at 37 °C than at 18 °C in shorter induction time (3 h vs 5 h), we decided to perform the rest of the experiments with membrane fragments obtained from cells grown at 37 °C induced for 3 h. Note that the apparent molecular weights of the different overexpressed fusion proteins corresponded quite well with the deduced amino acid sequence of the corresponding constructions: MBP + polylinker + lacZ alpha peptide: 50,843.27 Da; MBP + polylinker + full α -subunit from *Synechocystis*: 52,533.28 Da; MBP + polylinker + chimeric α -subunit I17 from *Synechocystis*: 50,753.30 Da. To better identify them, immunoblot analysis was done using a commercial monoclonal antibody against the MBP protein (Fig. 2c) or polyclonal antibodies against a synthetic peptide of the C-terminus α -subunit from *T. elongatus*. Thus the results clearly demonstrated that the overexpressed fusion proteins corresponded with the described Coomassie bands, as indicated with arrows in the Fig. 2b, 2c and 2d. The presence of some additional immunoreactive bands with close apparent molecular weights could most probably result from slightly different migration in the gel and/or different translation completeness of the

overexpressed proteins in the bacteria. We have observed a similar phenomenon using the same protein carrier (MBP) fused to the chloroplastic copper chaperone for the Cu/Zn superoxide dismutase, the CCS [30]. Note that no lower break-down products were detected in the western blot.

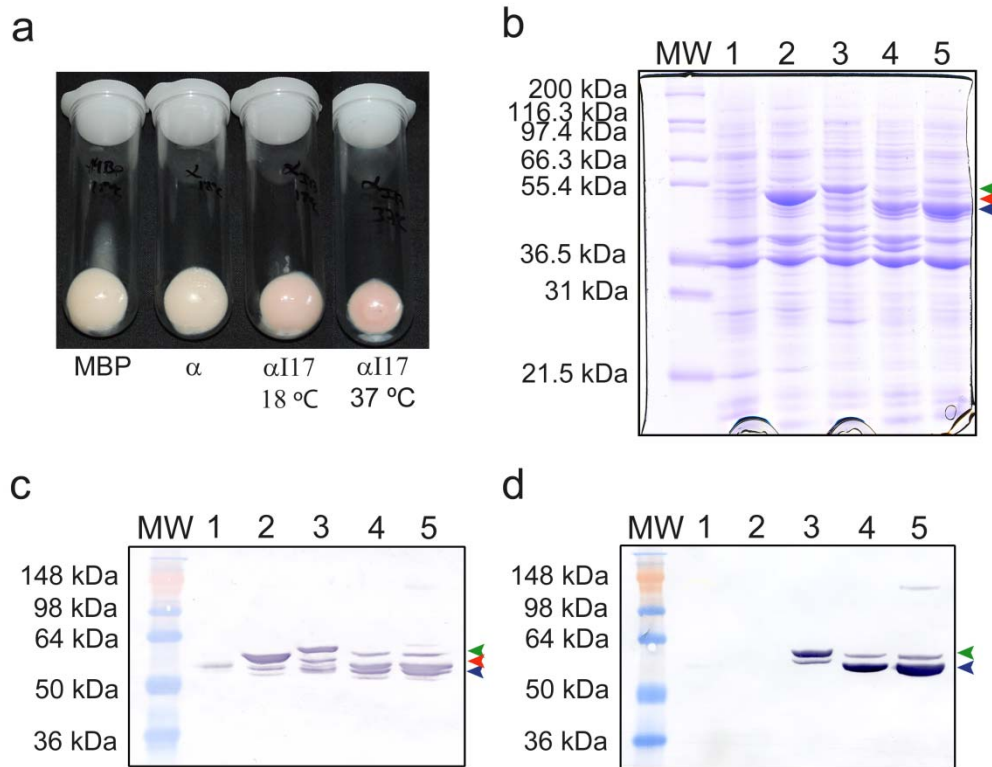


Fig.2: Overexpresion of fusion proteins. *a*, pellets of *E. coli* TB1 cultures: induced at 37 °C for 3 h containing only the MBP (MBP); induced at 18 °C for 5 h containing the MBP-full length *Ssuba* (α); induced at 18 °C for 5 h containing the MBP-chimeric *Ssuba*I17 (α I17 18 °C); induced at 37 °C for 3 h containing the MBP-chimeric *Ssuba*I17 (α I17 37 °C). *b*, SDS-PAGE [12% (w/v) acrylamide, 4 M urea] analysis of bacterial membrane fragments obtained from the pellets described in part *a*; *c*, Immunoblots with mouse monoclonal antiMBP antibody; *d*, Immunoblots with mouse antibodies against a synthetic peptide of the C-terminus α -subunit from *T. elongatus*. MW, molecular weight markers; lane 1, TB1 bacterial membrane fragments without transformation; lane 2, containing the MBP only (MBP-lacZ fusion protein of 50.8 kDa; red color arrow); lane

3, containing the MBP-full length *Ssuba* (fusion protein of 52.5 kDa; green color arrow); lane 4, containing the MBP-chimeric *SsubaI17* (fusion protein of 50.7 kDa; blue color arrow) at 18 °C; lane 5, containing the MBP-chimeric *SsubaI17* at 37 °C (fusion protein of 50.7 kDa; blue color arrow)

3.2. Release of the peripheral MBP protein

As explained in Materials and Methods, the constructions used in this study resulted necessarily in the presence of the MBP protein in the bacterial cytoplasmic space, as demonstrated unambiguously in previous works [21,24-26] using similar constructs. Thus the heme group of our chimeric Cyt *b₅₅₉* like structure has to be located necessarily towards the inner surface of the cytoplasmic membrane. Since the MBP protein is highly overexpressed in our bacterial cultures, it appeared in both soluble and membrane fractions [19,22]. Due to the lack of any clear membrane-spanning element, the MBP protein should be located on the surface as peripheral membrane protein. To prove it, cytoplasmic membranes were treated with NaBr or TRIS-HCl to release the non-integral membrane proteins. SDS-PAGE analysis demonstrated that such treatments indeed released a good part of the MBP protein content from the membranes in the soluble fraction (Fig. 3, lane 2). But a significant fraction of the MBP still remained with the membrane (Fig. 3, lane 3), which would correspond to proteins trapped inside the small membrane vesicle and fragments during the sonication process. Such a process would generate a mixture of small membrane vesicle with different surface orientation, and the NaBr or TRIS-HCl treatments would only release the peripheral proteins of the exposed surface.

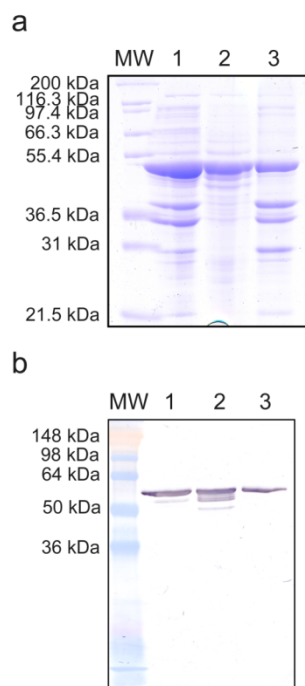


Fig.3: Membrane fragment treatment with NaBr. Membranes from bacterial cells containing only the MBP protein were treated with 2 M NaBr (similar results were obtained with TRIS-HCl). a, SDS-PAGE Coomassie brilliant blue protein staining; b, Immunoblots using a commercial monoclonal antibody against MBP protein. MW, molecular weight markers; lane 1, control membranes without treatment; lane 2, resultant supernatant after ultracentrifugation; lane 3, resultant pellet after ultracentrifugation

3.3. Electronic absorption spectra of the cytoplasmic membrane pellets

The degree of overexpression of the Cyt b_{559} like structure can be assessed by the intensity of the visible spectral bands at around 559 nm (α -band) or 530 nm (β -band) after chemical reduction with sodium dithionite. Membranes from control cells (only with MBP) or with full length α -subunit (MBP-Ssub α) showed much smaller spectral band intensities, due to intrinsic bacterial membrane cytochromes, compared to membranes from cells with the chimeric α -subunit (MBP-Ssub α I17) both grown at 18 °C or 37 °C (Fig. 4), being somewhat higher in the latter and according to the SDS-PAGE analysis (Fig. 2b, lanes 4 and 5).

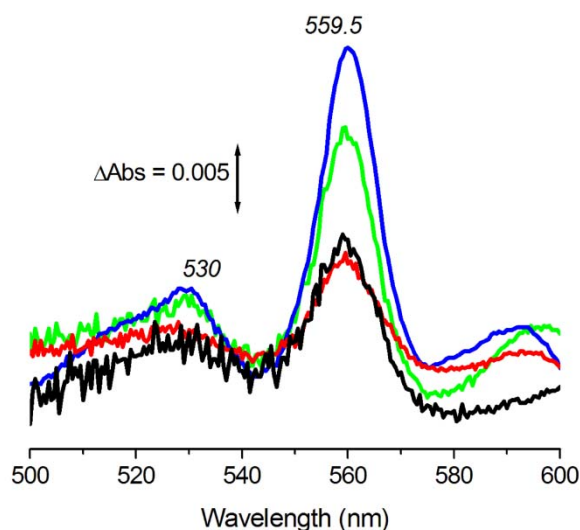


Fig.4: Redox differential absorption spectra of the different bacterial cytoplasmic membrane fragments within the 500-600 nm spectral range in the presence of 10 mM sodium dithionite. *a*, containing only the MBP (black color line); expressing the MBP-full length *Ssuba* (red color line); expressing the MBP-chimeric *SsubaI17* at 18 °C (green color line); expressing the MBP-chimeric *SsubaI17* at 37 °C (blue color line). Spectra were normalized to total protein content

3.4. Partial purification of the MBP-chimeric α -subunit

To even better identify the overexpressed chimeric protein as a Cyt b_{559} like structure, a partial purification was attempted using membrane solubilization with 1% (w/v) Triton X-100 of cytoplasmic membranes, previously treated with 50 mM TRIS-HCl, pH 8.0. The resultant supernatant after ultracentrifugation at 150,000 x g contained most of the overexpressed chimeric protein as shown by SDS-PAGE (Fig. 5a, lane 2; blue color arrow). Surprisingly, most of the other membrane proteins remained in the pellet (Fig. 5b, lane 3), indicating the high efficiency and selectivity of the Triton X-100 extraction. The supernatant depicted a visible absorption spectrum with maximum peaks at around 559 and 530 nm (Fig. 5b), which corresponded well with the spectrum of the native Cyt b_{559} . No spectral signals were observed from the resultant insoluble membrane pellet (Fig. 5b), and according to the SDS-PAGE analysis (Fig. 5a).

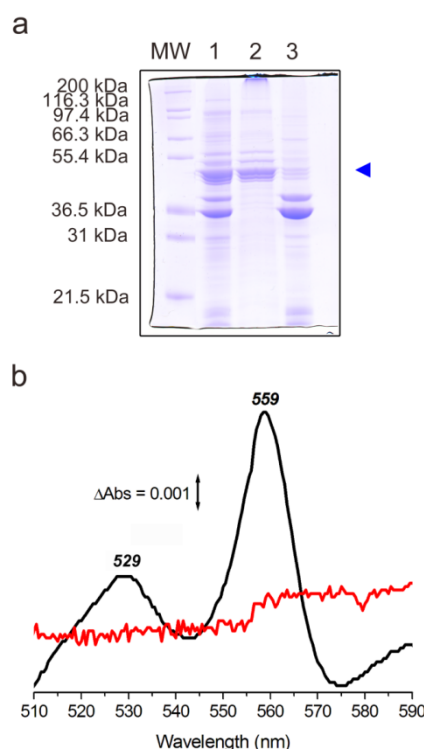


Fig.5: Partial purification of MBP-chimeric *SsubaI17* fusion protein by membrane solubilization with Triton X-100. *a*, SDS-PAGE [12% (w/v) acrylamide, 4 M urea]: MW, molecular weight markers; lane 1, cytoplasmic membrane fragments previously treated with 50 mM TRIS-HCL, pH 8.0; lane 2, resultant supernatant after ultracentrifugation of detergent solubilized extract; lane 3, resultant pellet after ultracentrifugation. The arrow indicates the MBP-chimeric *SsubaI17* fusion protein. *b*, redox differential absorption spectra of the supernatant (black color line) and the pellet resuspended in 50 mM HEPES, pH 7.5 (red color line)

3.5. Electron paramagnetic resonance spectra of the membranes

Since Fe^{3+} ion is a paramagnetic species, EPR is a sound technique to characterize the Cyt b_{559} present in the cytoplasmic membrane fragments. It is well established that the natural heterodimeric Cyt b_{559} is a low spin species with characteristic signals of $g_z = 3.0\text{-}2.9$, $g_y = 2.26$, and $g_x = 1.4$ [7,8,31,32]. Membrane preparation with the MBP protein alone was used as control, and showed almost undetectable EPR spectral signals [19]. The residual signals would correspond to some endogenous low spin hemoproteins present within the bacterial membrane (Fig. 6, MBP). When membranes with the chimeric fusion protein were used, the spectral features from low spin Cyt b_{559} were now apparent (Fig. 6, αI17). When the control was subtracted from this new spectrum (Fig. 4, $\alpha\text{I17-MBP}$) and the low spin region expanded (Inset), the presence of low spin

Cyt b_{559} was even more apparent. The signals in the spectrum correspond to g values $g_z = 2.96 \pm 0.03$, $g_y = 2.27 \pm 0.01$, and $g_x = 1.47 \pm 0.05$, fully compatible with a non-distorted Cyt b_{559} species. A signal at around $g = 4.3$ is due to non-heminic iron centers, and the feature at around $g = 6.0$ is due to high spin heminic centers. The latter is most probably due to the displacement of one histidine Fe axial ligand by other ligand [31], but the concentration of the high spin paramagnetic centers was very low compared to the low spin centers [19].

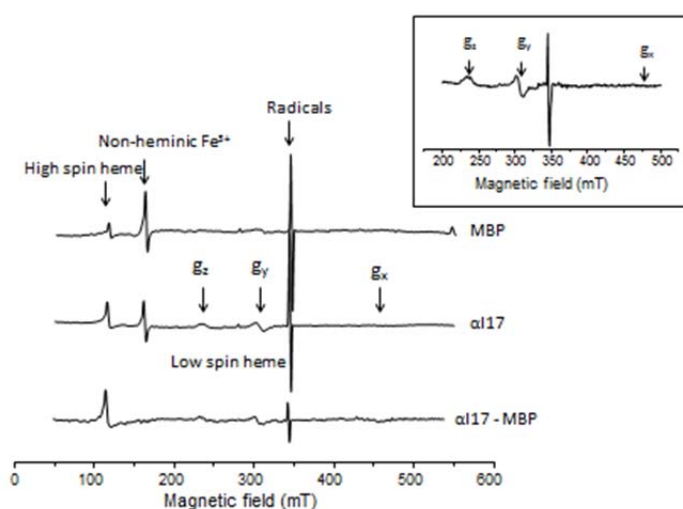


Fig.6: X-band EPR spectra of *E. coli* TB1 cytoplasmic membrane fragments containing MBP protein only, containing the chimeric homodimer ($\alpha I17$) Cyt b_{559} like structure, and the subtraction of the two spectra ($\alpha I17$ -MBP) (normalized to the $g = 4.3$ signal height). Spectral features corresponding to high spin heme, non-heminic iron, low spin heme and radicals are indicated. The inset shows a detail of the low spin features from the subtraction spectra that correspond to the Cyt b_{559} low spin features. For experimental conditions, see Materials and Methods section

3.6. Potentiometric titration of the membranes

The E_m of the *in vivo* reconstituted chimeric homodimeric Cyt b_{559} was measured by potentiometric reductive titrations at pH 6.5 (Fig. 7), as described in Materials and Methods. As a control, potentiometric reductive titrations of membrane fragments with MBP protein alone were carried out, and only one redox species with an E_m of about +67 mV was calculated, which would be due to endogenous cytochrome of the bacterial cytoplasmic membrane (Fig. 7). When membrane fragments containing the chimeric

MBP-Ssub α I17 were used, they exhibited two redox potentials species with E_m of +67 mV and +180 mV (Fig. 7). The lower E_m component would correspond again to the endogenous cytochrome and the new component with the higher E_m to the overexpressed and *in vivo* reconstitution of a chimeric homodimeric (α/α) Cyt b_{559} like structure. Note the good correlation between the ratio of the absorption changes of the chimeric and control membranes (Fig. 4, blue and black lines) and the changes corresponding to the +180 mV and +67 mV (Fig.7, black line) redox forms, in both cases the ratio was around 2.3-folds.

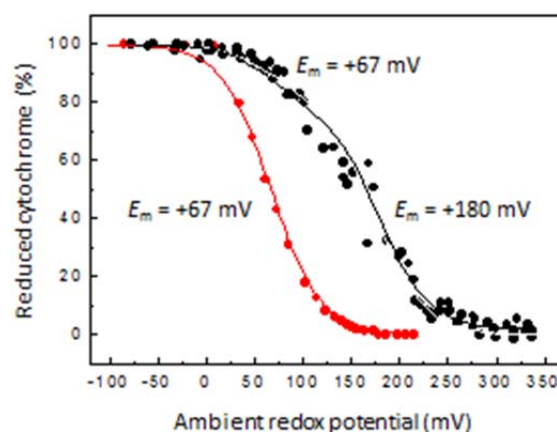


Fig.7: Reductive potentiometric titrations of the cytochromes present in *E. coli* TBI cytoplasmic membrane fragments. Redox titration was carried out in cytoplasmic membrane fragments containing either the MBP protein only (red) or the chimeric homodimer (α I17/ α I17) Cyt b_{559} like structure (black), as described in Materials and Methods. The figure represents the plot of the percentages of reduced cytochromes obtained from the absorbance differences at 559–570 nm versus ambient redox potentials from the two redox titrations. Solid curves represent the best fits of the Nernst equation to the experimental data in accordance with one-electron processes ($n = 1$) for one (red line) or two (black line) components. For other details see Materials and Methods

4. Discussion

In the present paper, we demonstrated that the full length α -subunit was unable to reconstitute a Cyt b_{559} like structure *in vivo* despite its incorporation and stabilization within the cytoplasmic membrane as shown by the SDS-PAGE analysis (Fig. 2b, lane

3). By contrast considering the high structural homology between both subunits, the situation with the full length β -subunit was completely different where the *in vivo* formation of a Cyt b_{559} like structure was indeed possible [19,21]. According to the structure of the natural heterodimeric (α/β) Cyt b_{559} from cyanobacteria (Figs. 1 and 8), the position of two amphipathic short α -helices of the N-terminus full length α -subunit on the cytoplasmic membrane surface may block the entry of the heme to coordinate the two histidines to mature the chromoprotein (Fig. 8, $\alpha\alpha$). After rid of the complete out-of-membrane N-terminus part of the α -subunit, the *in vivo* reconstitution of a chimeric homodimeric (α/α) Cyt b_{559} like structure was well possible, as shown by the brownish color of the cell culture pellet, SDS-PAGE, immunoblots, partial purification, and absorption and EPR spectra. We think the elimination of the protein steric hindrance on the surface of the membrane at the entry of the heme, would now allow the entrance of the heme group to coordinate to the two histidine ligands to mature a Cyt b_{559} like structure (Fig. 8, $\alpha I17\alpha I17$). This conclusion was highly reinforced by using two

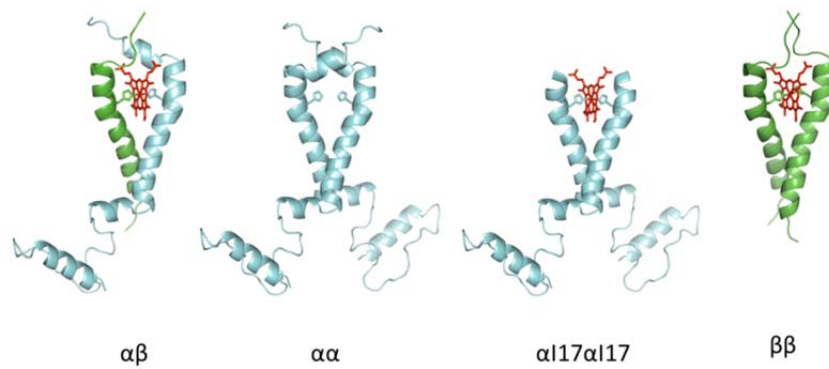


Fig.8: Ribbon representations of the different structures of Cyt b_{559} . $\alpha\beta$, natural Cyt b_{559} of the PSII core from *T. vulcanus* at 1.9 Å; $\alpha\alpha$, full length α -subunit homodimer; $\alpha I17\alpha I17$, chimeric α -subunit homodimeric Cyt b_{559} like structure; $\beta\beta$, full length β -subunit homodimeric Cyt b_{559} like structure

additional N-terminus chimerics, one without the first eight amino acids but preserving the entire short amphipathic α -helix (called Ssub α P9), and the other without the first twelve amino acids preserving about half of the short α -helix (called Ssub α I13) (Fig. 9). In the case of the Ssub α P9, the reconstitution of a Cyt b_{559} like structure was not possible as in the case of the full-length α -subunit, but in the case of the Ssub α I13 a somewhat partial reconstitution was indeed possible. This strongly indicates that the presence of two entire amphipathic α -helices may block the entry of the heme group and

interrupt the membrane-bound cytochrome maturation. In principle, protein steric hindrance could also occur between the two full length protein subunits at the N-terminus avoiding the dimerization of the full length α -subunit within the membrane. But this is very unlikely since the dimerization process is controlled by a minimum of six amino acid sequence around the conserved glycine (Gly34 in the case of *Synechocystis sp.* PC 6803) located in the α -helix towards the membrane periplasmic side [26].

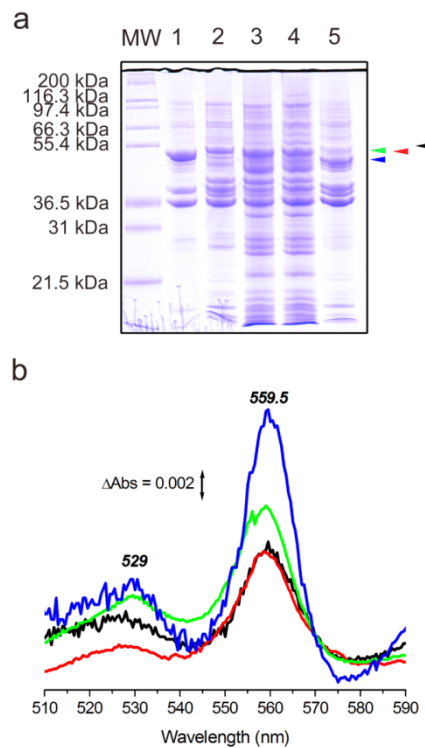


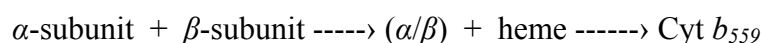
Fig.9: Overexpression of different chimeric and full length α -subunits at 18 °C. a, SDS-PAGE [12% (w/v) acrylamide, 4 M urea] analysis of bacterial membrane fragments: MW, molecular weight markers; lane 1, containing only the MBP (MBP-lacZ fusion protein of 50.8 kDa); lane 2, containing the MBP-full length Ssuba (fusion protein of 52.5 kDa; black color arrow); lane 3, containing the MBP- chimeric SsubaP9 (fusion protein of 51.7 kDa; green color arrow); lane 4, containing the MBP-chimeric SsubaI13 (fusion protein of 51.2 kDa; red color arrow); lane 5, containing the MBP-chimeric SsubaI17 (fusion protein of 50.7 kDa, blue color arrow). b, redox differential absorption spectra of the bacterial cytoplasmic membrane fragments within the 510-590 nm spectral range: containing MBP-full length Ssuba (black color line); the MBP-chimeric SsubaP9 (red color line); the MBP-chimeric SsubaI13 (green color line); the MBP-chimeric SsubaI17 (blue color line)

As mentioned in the section 3.1. of the Results, several attempts to overexpress the α -subunit without the MBP carrier were unsuccessful. In principle, one could think that the presence of a much bigger carrier protein compared to the α -subunit chimeric α I17 (42.5 vs 7.8 kDa) might interfere with a proper reconstitution of a Cyt b_{559} like structure. But this clearly seemed not to be the case in the present work. Indeed, the absorption spectra of the membranes overexpressing the chimeric fusion protein (Ssub α I17) identified a Cyt b_{559} like structure with the typical α and β spectral peaks at around 559 and 530 nm, respectively. The visible absorption spectra of the partially purified overexpressed chimeric fusion protein along with the EPR characteristics and the redox potentiometric titration nicely confirmed the nature of the reconstituted form as a Cyt b_{559} like structure. Whether the MBP protein carrier might interfere with the heme assembly in the case of the full length native α -subunit but not with the α -chimeric is very unlikely since the MBP should be closer to the heme niche in the chimeric compared to the full length α -subunit due to the lack of the N-terminus in the former. Moreover, the use of this specific plasmid construct with the MBP carrier [21,24-26] assured that the reconstituted Cyt b_{559} like structures have a homogeneous orientation with the heme group towards the inner surface of the cytoplasmic bacterial membrane.

The E_m of about +30-50 mV, observed for the isolated natural Cyt b_{559} [10,32] or the reconstituted form in solution [19], increased up to +180 mV in the *in vivo* reconstitution presented in this work, most probably due to the higher hydrophobicity imposed by the membrane lipid and the rearrangement of the heme pocket after its insertion within the membrane. However, in both the homodimeric (β/β) [19] and chimeric homodimeric (α I17/ α I17) (present work) forms, the redox potentials were significantly lower than +300-400 mV, as observed for the HP form of the natural heterodimeric (α/β) Cyt b_{559} within the photosynthetic membrane [8,11,15]. The heme pocket generated in the *in vivo* homodimeric reconstitutions (Fig.8, α I17/ α I17 and β/β) might be too open, increasing the exposition to the surrounding solvent and thus reducing its hydrophobicity and the redox potential of the heme [33,34]. This situation might be overcome by the presence of an amphipathic α -helix that shields the heme niche from outside as in the case of the natural heterodimer (α/β).

Furthermore, our data suggest that the photosynthetic natural Cyt b_{559} assembly and maturation would most probably occur by a three step model as demonstrated for

the (β/β) homodimer in [35,36]. First, the polypeptides enter the membrane matrix; second, an α -helix interacts with and recognizes another α -helix at around the conserved glycine and phenylalanine residues near the thylakoid lumen surface to make a (α/β) heterodimer; and third, the heme group is incorporated to coordinate the two histidines and form a mature holoprotein. According to our data and other results [35,36], the heme group seems not to be necessary for the incorporation, recognition and stable assembly of the protein complex subunits. The consecutively maturation steps would be as the following:



However, the proposed model cannot be generalized to other more complex cytochromes and chromoproteins and each protein has to be studied separately. For instance, in the case of the retinal, the sole chromophore of a more complex and well-known membrane-bound protein as bacteriorhodopsin with seven transmembrane α -helices, it was also no necessary for the formation of the protein folding structure within the membrane [37]. But in the case of the photosynthetic light-harvesting complex (LHCII), the chromophores, chlorophylls and carotenoids, were indeed necessary for the polypeptide folding and protein complex maturation [38,39].

Finally, in the present work we have tried to answer the question of why the full length α -subunit in contrast to the full length β -subunit, was unable to *in vivo* reconstitution of a Cyt b_{559} like structure, despite the high structural homology between these two subunits. Our hypothesis that a structural steric hindrance due to the presence of an out-of-membrane amphipathic α -helix was the cause of lack of cytochrome maturation, is strongly supported by the results. The presence of that amphipathic α -helix might also affect the redox potential of the heme niche. The present structural work is even more relevant considering the scarce information available on integral membrane protein maturation as compared to the soluble ones. Whether this chimeric structure can be actually synthesized and assembled in a photosynthetic system and, more important, whether these homodimeric structures (chimeric $\alpha I17/\alpha I17$; β/β) could be functional photosynthetically remain to be proved. An on-going project in our laboratory is trying to respond to these interesting questions.

Acknowledgements

We thank P. Lorente for technical assistance. This work was supported by Grants from the Spanish Ministry of Economy and Competitiveness (MINECO) (AGL2011-23574, MAT2011-23861, BIO2012-35271, BFU2010-16297), the Aragón Regional Government (E33 and B18), and the Andalucía Regional Government (PADI BIO-022). All these Grants were partially financed by the EU FEDER Program.

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